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Fertilization (N or S) and Inoculation Effects on California Native Annual Range Legumes

Final Report

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INTRODUCTION

The importance of legumes as a forage crop is a result of the ability of these plants to enter into symbiotic association with N-fixing bacteria of the genus Rhizobium. Significant quantities of N may be fixed by range legumes. At the San Joaquin Experimental Range (SJER), in the annual grass rangelands of the central California Sierran foothills, an estimated 50 kg N ha^{-1} was fixed by legumes in the 1972-1973 growing season (12). When legumes constitute a large component of the forage crop, as in that year, forage quality is improved for both livestock and wildlife (2). Thus, economically sound management practices which increase legume growth are recommended (1, 22). Sulfur fertilization increases nodulation and growth of legumes at SJER (1,9,11) as at other grassland sites (12), although its effects on nitrogen fixation activities of native legumes have not been tested.

In this study, the effects of S fertilization on nitrogen fixation (acetylene reduction) and growth response in pot culture of California native annual legumes were determined. The test plants were five important SJER forage legumes--Trifolium microcephalum, T. ciliolatum, T. variegatum, Lotus purshianus and Lupinus bicolor. Soil in which legumes had not recently grown was used and plants were inoculated with single strains of known effective indigenous rhizobia to reduce heterogeneity of plant responses (Mark Poth, personal communication). Uninoculated plants were also included to determine the effects of inoculation. Nitrogen-fertilized plants were included as controls to determine the extent to which nitrogen fixation met the N needs of the plants.

MATERIALS AND METHODS

Preparation of inocula.

Field-collected seeds of *T. microcephalum*, *T. ciliolatum*, *T. variegatum*, *Lotus purshianus* and *Lupinus bicolor* were sown in soil from sites in which each plant species had been observed growing in the previous season. When these greenhouse grown plants were near maturity, leghaemoglobin-containing nodules from large, healthy plants were used for isolation of rhizobia on Yeast Mannitol agar (YM; 5). Tube infection tests (9) with acetylene reduction assays (see below) were used to verify the effectiveness of isolates on the homologous hosts. Inocula were grown 72 hr with shaking at 30°C in YM broth, washed once and resuspended in 0.01 M phosphate buffer, pH 7.0, to a final cell density of 2-6 x 10^9 colony forming units ml^{-1}.

Plant cultivation.

Field-collected seeds of the five plant species were sown in coarse, sandy loam of the Ahwanee series (Mollic Halplustalfs). These soils are marginal in N and S content, but adequate in P (12, 21). Soil for this test was obtained from a storage heap at the SJER in which legumes had not been observed growing for eight years. All seeds were scarified mechanically, covered with 82°C water and soaked overnight before planting. Ten seeds were planted in each pot. *Lotus purshianus* was grown in 20 cm diameter plastic pots; the other species were grown in 15 cm pots.

Fertilization was begun 28 d after seeding. Sulfur-fertilized pots received 20 ml of 0.19 M $CaSO_4.2H_2O$ (65 kg S ha^{-1}). Nitrogen-fertilized pots received a total of 8 ml of 0.29 M NH_4NO_3 applied over a period of one week to previously moistened soil (35 kg N ha^{-1}).

Inoculated pots received 50 ml of bacterial suspension 28 d post-seeding; control pots received 50 ml of autoclaved rhizobial suspension. All species had formed the first true leaves at the time of inoculation. The three fertilizer levels (N, S and unfertilized) were crossed with the inoculation levels (plus and minus inoculation) with three replicates per cell. Triplicate unplanted pots of soil also received each treatment combination and were incubated and watered with the pots containing plants.

All pots were placed in a single growth chamber with day length and temperatures regulated to approximate field conditions during the growing season. A twelve hour day/night cycle was used until 90 d post-seeding with 14 hr days thereafter. Irradiance was 160 W m^{-2}. Temperatures were 21°C / 7°C (day/night) until 33 d post-seeding, 21°C / 10°C until 62 d post-seeding, followed by 21°C / 12°C until the termination of the experiment (121 d). Pots were watered with deionized water. At 111 d post-seeding, all plants were allowed to dry until wilted.

Acetylene reduction assay.

Plants were watered the day before acetylene reduction activity (ARA;19) was assayed. The assay was carried out in polycarbonate jars (Nalgene). Lids fitted with serum stoppers were sealed to the jars with Vaseline and clamped with rubber tubing. After removal of 10% of the gas volume in the jar, CaC_2 was used to generate an equivalent amount of acetylene within the jar. Samples (0.5 ml) were assayed for C_2H_4 at 60, 90 and 120 min after acetylene generation using a Carle 310 gas chromatograph equipped with a flame ionization detector and a 0.32 cm x 1.86 m Porapak N (80/100 mesh) column. The carrier gas was He at 55 ml min^{-1}. Oven temperature was 50°C.

Plant and soil analyses.

Shoot and root biomass of plants harvested at the termination of the experiment were determined after drying overnight at 50°C. Nitrogen contents of plant shoots (11) and soil (7, 8) were determined by Kjeldahl analyses.

Lotus purshianus nodules in each pot were counted under a dissecting microscope at 30X magnification. Lupinus bicolor nodules were removed from weighed roots and weighed separately. No attempt was made to quantify the numerous small nodules appearing on Trifolium.

Statistical analyses.

Two way analyses of variance and covariance with the number of plants per pot as the covariate were performed using BMDP4V (4) on the California State University, Fresno Cyber 720 computer. Single degree of freedom contrasts were used for planned comparisons of treatment effects (effects of fertilization with N or S within inoculation level and effects of inoculation within fertilization level). Bartlett's test of homogeneity of variance (18) was carried out using BMDP9D. Analysis of variance for repeated measures (BMDP4V) was used to analyze ARA and the least significant difference (18) was used to determine treatment effects at each assay date.

RESULTS

Replication.

Some reduction in replication was necessary due to heterogeneity of pots (Table 1). Three pots of Lupinus bicolor were omitted. Two of these contained beetle larvae (Cebrionidae, probably Scaptolenus sp.; D. Burdick, personal communication) feeding on the plant roots and one plant was depauperate due to loss of soil from the pot. Several pots contained soil which was especially sandy in texture. These were also omitted from the analyses as soil texture appeared to affect plant shoot weight and increased the heterogeneity of plant responses to the treatments.

Plant phenology.

The Trifolium species seemed to mature normally in the environmental chamber. Lotus purshianus was slow to germinate under the artificial conditions, but was the first of the species to bloom. This species was still blooming at the termination of the experiment, even though a few basal leaves had dropped, indicating the onset of senescence. Lupinus bicolor did not grow well in the environmental chamber. The plants were tall and spindly with sparse blooms compared to the smaller, compact plants found on the rangeland.

Unfertilized, inoculated T. macrocephalum bloomed before other plants of this species (Table 1). T. ciliolatum plants all bloomed at the same time, regardless of treatment. T. variegatum bloomed one week earlier in unfertilized, uninoculated pots than in the remaining pots. Lotus purshianus inoculated plants bloomed earlier than uninoculated plants, and N-fertilization speeded blooming. Lupinus bicolor bloomed first in N-fertilized, inoculated pots, but inoculation delayed blooming four weeks in S-fertilized and one week in unfertilized pots.

Acetylene reduction activity.

No ARA was detected in unplanted pots of soil. Acetylene reduction activity was linear over the time period used for rate calculation (60-120 min) and was not affected by the number of plants per pot. In all species, ARA ceased after plants had dried to the wilting point 83 d post-inoculation, even though most of the plants appeared to recover completely from water stress.

Sulfur fertilization affected ARA only in T. variegatum and Lotus purshianus (Figure 1). In the clover, it appeared to prevent the increase in ARA due to inoculation at the last sampling date. In Lotus purshianus, S fertilization increased the rate at which ARA developed in inoculated plants, but had little effect on maximum activity.

Combined N fertilization and inoculation delayed the onset of ARA in T. microcephalum, T. variegatum, Lotus purshianus and Lupinus bicolor. Fertilizer N also decreased ARA of uninoculated T. ciliolatum and Lupinus bicolor, but increased ARA of uninoculated T. variegatum.

The effects of inoculation on ARA varied considerably with the legume species. Inoculation increased ARA of unfertilized T. microcephalum and T. variegatum, of both unfertilized and S-fertilized Lotus purshianus and of S-fertilized Lupinus bicolor. Inoculation decreased ARA of T. ciliolatum regardless of fertilization level and decreased ARA of N-fertilized T. variegatum. As combined N fertilization and inoculation decreased and delayed ARA, treatment interactions were observed for those species showing strong increases in ARA due to inoculation in pots not receiving N (T. microcephalum, $p < 0.04$; T. variegatum, $p < 0.001$; Lotus purshianus, $p < 0.002$).

Biomass and N content.

The five plant species differed in their growth responses to fertilization and inoculation (Table 2). No heterogeneity of variance was detected. Treatment

effects on root weight generally corresponded to effects on shoot weight, although inoculation increased the ratio of root weight to shoot weight (data not shown) in T. microcephalum ($p < 0.003$) and in unfertilized Lotus purshianus ($p < 0.05$). Treatment effects on total shoot N were similar to those on ARA in plants which were not given fertilizer N. The number of plants per pot did not generally affect growth parameters; exceptions are noted below.

Shoot characteristics (dry weight and N content) of T. microcephalum were not affected by fertilization or inoculation, in spite of the increased ARA at later assay dates due to inoculation. Inoculation did increase root weight of this species, particularly in fertilized pots. Nitrogen fertilization also increased root weight of inoculated plants. Root weight of T. microcephalum was higher in pots containing more plants (p of plant number as covariate < 0.03).

Inoculation decreased shoot weight of T. ciliolatum in unfertilized, but not in fertilized pots, resulting in apparent increases in shoot weight due to N or S fertilization of inoculated plants. Neither fertilization nor inoculation affected total shoot N. A significant increase in root weight was observed only for S-fertilized, inoculated plants. Treatment interactions were observed for both shoot ($p < 0.03$) and root ($p < 0.04$) dry weights.

In T. variegatum, in contrast to T. ciliolatum, inoculation increased shoot weight of unfertilized, but not of fertilized plants, resulting in apparent decreases in shoot weight due to N or S fertilization of inoculated plants. A treatment interaction ($p < 0.02$) was observed. Total shoot N reflected response in shoot weight, again with an interaction between fertilization and inoculation ($p < 0.03$). Inoculation also increased root weight of unfertilized plants over root weight of inoculated, S-fertilized plants.

Both N and S fertilization increased shoot weight of inoculated Lotus purshianus. Inoculation increased shoot weight in N-fertilized plants of this species, resulting in a treatment interaction ($p < 0.01$). Like T. variegatum,

and unlike T. ciliolatum, treatment effects on Lotus purshianus shoot weight were reflected in increased total N content. However, the increases in total shoot N due to N or S fertilization and inoculation were additive. Shoot weight, but not total shoot N, was higher in pots containing more plants (p of plant number as covariant < 0.03). Root weight and nodule number, in contrast to shoot weight, were not increased by sulfur fertilization, but only by combined N-fertilization and inoculation. One N-fertilized, inoculated pot contained over 7000 nodules.

Soil N content.

At the termination of the experiment, residual total soil N of N-fertilized pots remained higher than that of unfertilized pots receiving the same planting and inoculation treatment (Table 3). Sulfur fertilization increased soil N of inoculated pots of unplanted soil and of the clovers. Sulfur also increased residual soil N of pots which had contained uninoculated Lotus purshianus. Inoculation decreased final soil total N of N-fertilized pots in which T. variegatum and Lotus purshianus had grown, and of unfertilized pots in which T. ciliolatum had grown, but increased N content of S-fertilized unplanted soil and S-fertilized soil in which T. variegatum had grown.

DISCUSSION

This study was designed to allow internal comparisons of the influence of S fertilization on ARA of native legumes rather than to obtain absolute measurements of nitrogen fixation. Particular caution must be used in extrapolating the results to field conditions, as the plant phenological characteristics were not identical to those observed in the field. Lotus purshiaus bloomed early in the environmental chamber, while Lupinus bicolor was slow to bloom. Under field conditions, Lupinus bicolor matures earliest, followed by T. microcephalum, T. ciliolatum and T. variegatum, with Lotus purshianus often maturing as late as August if late spring rains have been received.

The results of this study are not directly comparable with those obtained using different plant incubation conditions and assay systems. Vaughn and Jones (20), working with native clovers, found that ARA peaked near flowering time. Of the two species for which we obtained peak ARA values, only Lotus purshianus followed this pattern. There was little relationship between blooming date and peak ARA in T. microcephalum. The treatments applied in this study affected blooming date of all species except T. ciliolatum. Treatment combinations which resulted in high shoot weights or N contents at the termination of the experiment generally speeded blooming. In T. variegatum, however, uninoculated, unfertilized plants bloomed first, even though they were lower in ARA, shoot weight and shoot N than inoculated, unfertilized plants.

Vaughn and Jones (20) found that the peak ARA of T. microcephalum was nearly twice that of T. ciliolatum and T. variegatum. We found inoculated, unfertilized T. variegatum to be the most active of the three clovers. These differences could be attributed to differences in the plant genotypes, rhizobia or plant growth conditions.

One apparent effect of growth conditions observed in this study was the cessation of ARA when mature or nearly mature plants were subjected to moisture stress. Although most of the plants appeared to recover completely after reaching the wilting point 83 d post-inoculation, no ARA was detected at this time. Nitrogenase activity of many annual legumes declines as the plants begin seed production (17). In these range legumes, moisture stress may serve as a signal to redirect photosynthate to seed maturation rather than to nitrogen fixation.

Field experiments at the SJER have shown an increase in legume growth and nodulation due to S fertilization (1,10,12). In spite of its demonstrated value in the field, S fertilization had little effect on ARA of native legumes in pot culture. Plant growth also showed little response to added S. Only in inoculated Lotus purshianus were increases in shoot weight and shoot N clearly attributable to S fertilization. These results are at variance with a field study at SJER in which S fertilization as a comparable rate (65 kg ha^{-1}) increased shoot weights of native legumes at the end of winter growth from 60% for T. ciliolatum to 750% for Lupinus bicolor (10). The dramatic increase in legume growth observed in S-fertilized field soils may be more than a simple response to S deficiency. Applied S may stimulate early growth of legumes, enabling them to compete more effectively with grasses.

The results of this study are generally in agreement with field studies in which nitrogenous fertilizers reduce legume success by increasing growth of grasses (12). Legume shoot weights and N contents of most species were not increased by added N, indicating that nitrogen fixation was adequate to meet plant nitrogen requirements. Only Lotus purshianus exhibited a positive growth response to fertilizer N.

As in other legumes (6), fertilizer N repressed ARA of the species included in this study. This effect was most pronounced in inoculated pots of

T. microcephalum, T. variegatum and Lotus purshianus. The extent to which the legumes utilized the added N cannot be determined from this study. Residual soil total N at the termination of the experiment remained higher in N-fertilized pots, but the form of N is not known and it may have been unavailable to the plants. We cannot explain why S fertilization of inoculated pots increased residual N content of unplanted soil and of soil in which all the clover species had grown.

Inoculated plants were included in this study primarily to decrease the heterogeneity of plant growth responses. However, no difference in variability was detected between inoculated and uninoculated plants. Due to the significant interactions observed between inoculation and fertilization, both inoculated and uninoculated plants were included in the analyses. All plants were nodulated and exhibited ARA. While Lupinus bicolor nodule weights were comparable to those produced in the field (10), Lotus purshianus nodule nubmers were 20-80 times as high as produced under field conditions (10, personal observation). Although the soil used in this experiment had not grown legumes for eight years, rhizobia can survive in soil for as long as 10 to 125 years (13). Therefore, sufficient rhizobia may well have been present to allow nodulation to occur in the absence of inoculum.

Since all plants were incubated in a single growth chamber, cross-contamination of uninoculated pots from inoculated pots cannot be ruled out. Inoculation decreased ARA of T. ciliolatum, suggesting that the inoculum strain was less effective than the soil rhizobia forming nodules in uninoculated pots. Protein (14, 16) and plasmid (3, 15) gel electrophoresis techniques are currently being adapted to allow the indigenous rhizobia to be distinguished in future studies.

Combined inoculation and N-fertilization delayed the development of ARA, even in those species showing a positive response to inoculation in pots not

receiving N. The large inocula added at the time of fertilization may have interfered with the formation of effective nodules. It is conceivable that the inoculum strains were more sensitive to repression by combined N than were the soil rhizobia, but it seems unlikely that the strains selected would consistently behave in this fashion.

We have isolated several hundred indigenous rhizobia in order to further characterize their interactions with native legumes. We are determining diversity, host specificity and effectiveness of these bacteria on their homologous hosts. The effects of environmental conditions on the legume-rhizobium interactions will also be determined. These studies are preliminary to investigations of the factors influencing persistence and competitive success of rhizobia and of the role of plasmids in rhizobial evolution.

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Table 1. Replication[1], number of plants per pot and blooming date[2] of native annual legumes.

Species / Property	Treatment					
	N + Inoc.	N	S + Inoc.	S	Unfert. Inoc.	Unfert.
T. microcephalum						
Replicates	3	3	3	2	3	3
Plants pot^{-1}	2.7	3.7	3.0	4.5	3.7	2.7
Blooming date	72	72	72	72	65	72
T. ciliolatum						
Replicates	3	3	2	2	3	3
Plants pot^{-1}	5.0	3.0	2.0	3.0	2.3	3.0
Blooming date	86	86	86	86	86	86
T. variegatum						
Replicates	3	3	3	3	3	3
Plants pot^{-1}	3.0	8.7	6.3	5.3	5.0	4.7
Blooming date	72	72	72	72	72	65
Lotus purshianus						
Replicates	3	3	2	3	3	2
Plants pot^{-1}	5.0	4.6	3.5	6.0	4.0	7.0
Blooming date	45	50	50	57	50	57
Lupinus bicolor						
Replicates	3	2	2	2	3	3
Plants pot^{-1}	2.7	2.5	4.0	2.5	4.7	2.0
Blooming date	65	80	99	72	86	80

[1]Number of pots

[2]Days post-inoculation when first open flowers were observed

Table 2. Fertilization[1] (N or S) and inoculation[2] effects on mean[3] shoot dry wt (g), shoot total N (mg) and dry wt (g) of native annual legumes.

Planting	Treatment						
	N + Inoc.	N	N + Inoc.	S	Unfert. + Inoc.	Unfert.	CV (%)
microcephalum							
Shoot weight	9.62	8.2	9.5	7.6	10.0	8.96	23.6
Shoot N (mg)	211	205	231	183	229	210	25.2
Root wt (g)	5.91^{f,I}	3.04	5.80	2.22	4.53	2.99	28.4
T. ciliolatum							
Shoot wt.	11.6^{f}	10.3	12.5^{f}	9.6	7.83^{i}	11.6	16.9
Shoot N	256	223	282	210	193	249	19.8
Root wt	2.10	1.41	3.42^{F,i}	1.52	1.18	2.06	38.0
T. variegatum							
Shoot wt.	9.45^{F}	12.3	9.25^{F}	10.8	14.1^{i}	10.7	15.0
Shoot N	258^{F}	317	265^{f}	282	371^{i}	276	15.1
Root wt	1.84	1.93	1.23^{f}	1.34	2.95^{i}	1.18	45.1
Lotus purshianus							
Shoot wt	20.2^{F,I}	13.0	14.7^{f}	12.0	11.2	11.3	10.5
Shoot N	306^{F,I}	238^{F}	292^{F,I}	214	232^{I}	193	5.44
Root wt	14.8^{F,I}	3.7	10.0	6.4	7.3	4.0	34.6
Nodule	4200^{f,i}	1540	2700	1780	1470	1439	62.5
Lupinus bicolor							
Shoot wt	5.7	3.8	7.97	7.52	6.1	6.84	25.4
Shoot N	122	86^{f}	152	177	140	161	21.9
Root wt	0.26	0.34	1.40	1.10	1.11	1.27	54.0
Nodule	50	45	100	125	107	110	50.5

[1] Differences from unfertilized pots at same inoculation level denoted by F($p < 0.05$) or F($p < 0.01$).

[2] Differences from uninoculated pots at same fertilization level denoted by i($p < 0.05$) or I($p < 0.01$).

[3] Replication as in Table 1.

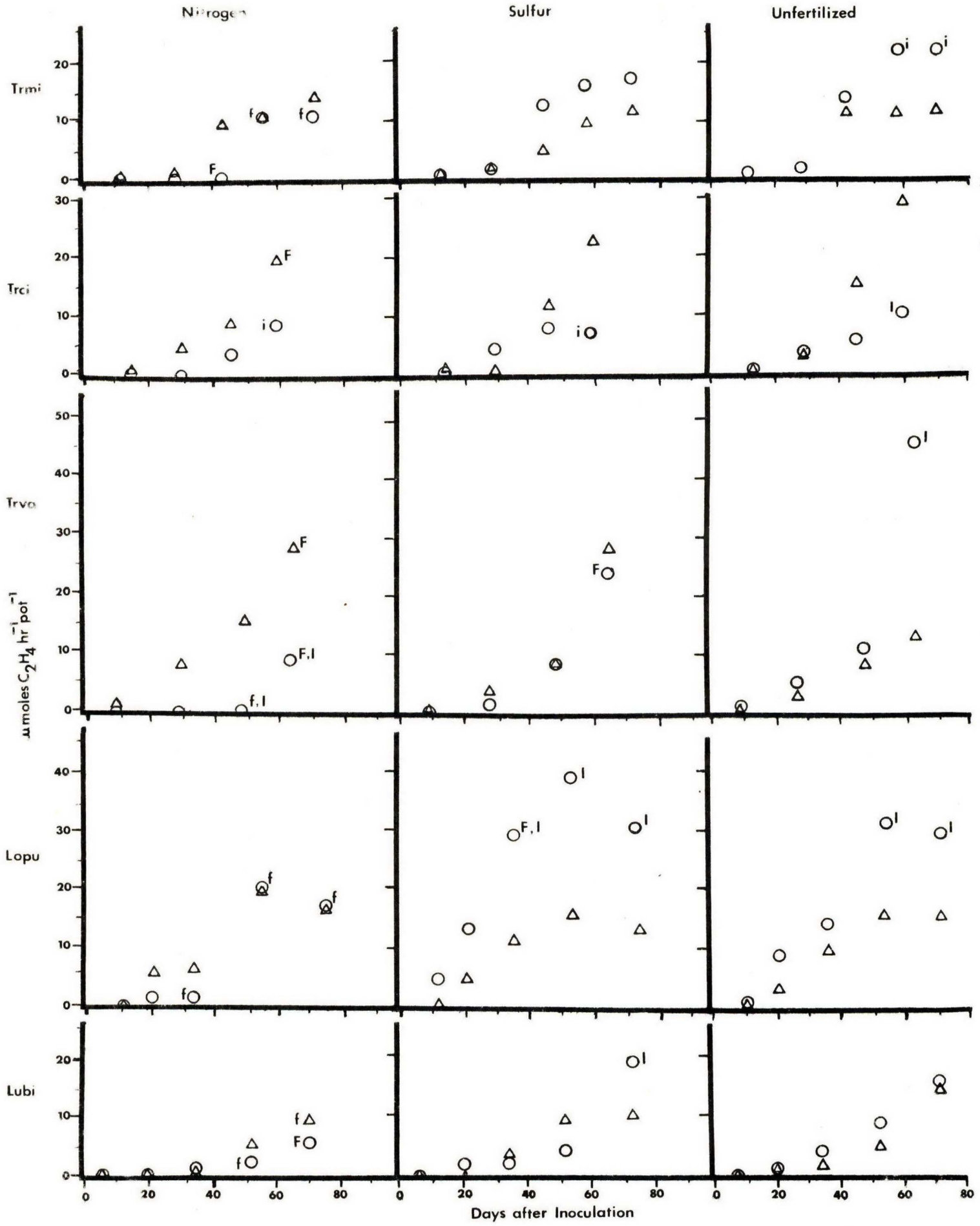
Table 3. Effects of fertilization[1] (N or S) and inoculation[2] on mean[3] residual total soil N (mg g^{-1}) after growth of native annual legumes.

Planting	Treatment						CV (%)
	N + Inoc.	N	S + Inoc.	S	Unfert. + Inoc.	Unfert.	
Unplanted	974^{F}	916^{F}	888^{F,J}	701	681	738	7.33
T. microcephalum	916^{F}	849^{f}	865^{F}	817	699	745	6.22
T. ciliolatum	854^{f,i}	990^{f}	882^{F}	868	692^{i}	852	8.88
T. variegatum	779^{f,I}	935^{F}	920^{F,i}	797	644	733	7.56
Lotus purshianus	773^{I}	1080^{F}	780	813^{F}	632	609	10.60
Lupinus bicolor	908^{F}	1010^{F}	723	836	664	758	7.83

[1], [2]As in Table 2.

[3]Replication as in Table 1; three replicates per cell for unplanted pots.

Figure 1. Effects of fertilization[1] (S or N) and inoculation[2] on ARA[3] of native annual legumes.


Nitrogen
Sulfur
Unfertilized
Trmi
Trci
Trvo
Lopu
Lubi
μmoles C_2H_4 hr^{-1} pot^{-1}
Days after Inoculation

[1] Differences from unfertilized pots at the same time and inoculation level are denoted by f($p < 0.05$) or F($p < 0.01$).

[2] ○, Inoculated; △, Uninoculated. Differences from uninoculated pots at the same time and fertilization level are noted by i($p < 0.05$) or I($p < 0.01$).

[3] Replication as in Table 1.

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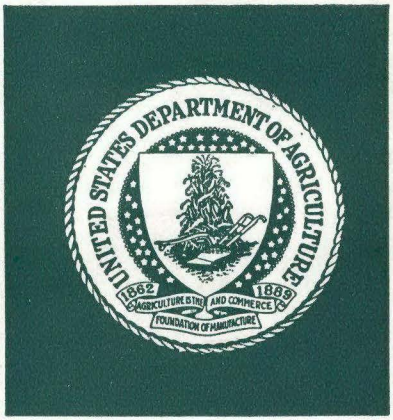

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